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Gap junctional intercellular communication has been linked to the regulation of cell proliferation and differentiation. Since most normal mammalian cells have functional gap junctions while most malignant cells do not, it has been hypothesized that the carcinogenic process involves the inhibition of this important biological process. Using several in vitro assays (metabolic cooperation; Fluorescent Recovery After Photobleaching or "FRAP"; scrape-loading/dye transfer; and the cell mat assay), we have examined the effects of various oncogenes, chemical tumor promoters, and growth factors on gap junction function. Natural products (phorbol esters, teleocidin), drugs (phenobarbital), food additives (saccharin), solvents (heptanol), pollutants (PCBs, PBBs), pesticides and herbicides (DDT, 2,3,5-T), nutritional factors (unsaturated fatty acids), growth factors (EGF, TGF-B), metabolic byproducts (H_2O_2, cholesterol epoxides), oncogenes (src, ras), cigarette tar condensates, heavy metals (mercuric chloride), neurotoxins (dieldrin) and neurotransmitters (acetylcholine) have been shown to modulate gap junctional communication. These observations suggest a possible integrativehypothesis linking oncogenes, which act as growth factors, with chemical tumor promoters which act as growth factors, and with growth factors,

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Chapter 12

Chemical Tumor Promoters, Oncogenes and Growth Factors: Modulators of Gap Junctional Intercellular Communication

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ABSTRACT

Gap junctional intercellular communication has been linked to the regulation of cell proliferation and differentiation. Since most normal mammalian cells have functional gap junctions while most malignant cells do not, it has been hypothesized that the carcinogenic process involves the inhibition of this important biological process. Using several *in vitro* assays (metabolic cooperation; Fluorescent Recovery After Photobleaching or "FRAP"; scrape-loading/dye transfer; and the cell mat assay), we have examined the effects of various oncogenes, chemical tumor promoters, and growth factors on gap junction function. Natural products (phorbol esters, teleocidin), drugs (phenobarbital), food additives (saccharin), solvents (heptanol), pollutants (PCBs, PBBs), pesticides and herbicides (DDT, 2,3,5-T), nutritional factors (unsaturated fatty acids), growth factors (EGF, TGF-B), metabolic byproducts (H_2O_2, cholesterol epoxides), oncogenes (src, ras), cigarette tar condensates, heavy metals (mercuric chloride), neurotoxins (dieldrin) and neurotransmitters (acetylcholine) have been shown to modulate gap junctional communication. These observations suggest a possible integrative hypothesis linking oncogenes, which act as growth factors, with chemical tumor promoters which act as growth factors, and with growth factors, which act as tumor promoters; namely via their common effect and inhibition of gap junctional communication.

"What clearly lies ahead is an era of research on intercellular communication at both local and systematic levels. The balance between proliferation and differentia-

tion must be examined at the molecular level, with emphasis on the interaction between growth factors, growth inhibitors, and their receptors and ultimate targets." (V.R. Potter [1])

INTERCELLULAR COMMUNICATION: CELLULAR BASIS FOR HOMEOSTASIS

The phenomenon of intercellular communication exists in all metazoans and is the means by which homeostatic control between and among various organs can be mediated (2). The transfer of ions and molecules between like or unlike cells can occur as systemic communication, by which substances enter the blood stream and are widely distributed within the body or as local and contact-dependent, by which ions and small molecules are transported via a membrane structure, the gap junction (Fig. 12-1) (3,4).

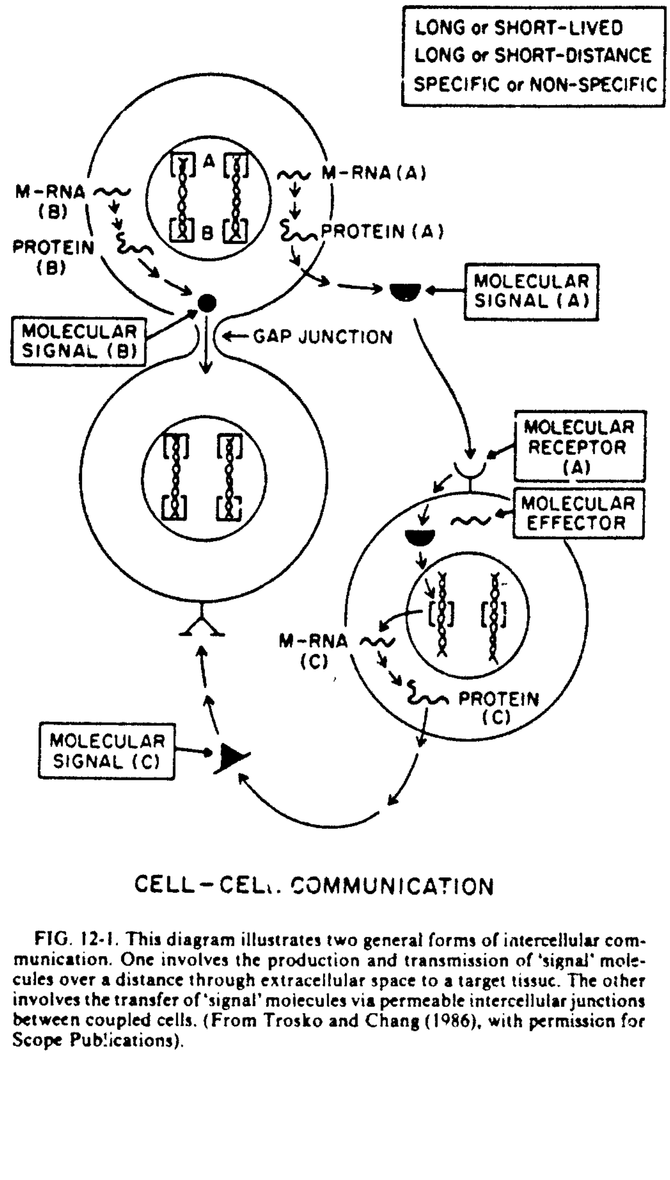

FIG. 12-1. This diagram illustrates two general forms of intercellular communication. One involves the production and transmission of 'signal' molecules over a distance through extracellular space to a target tissue. The other involves the transfer of 'signal' molecules via permeable intercellular junctions between coupled cells. (From Trosko and Chang (1986), with permission for Scope Publications).

For the purpose of this analysis, it must be recognized that (a) the two forms of intercellular communication are modulated by both environmental triggers, as well as genetic/developmental factors; and (b) they seem to be tightly coordinated, in that they can influence one another (5). Consequently, assuming the importance in maintaining homeostasis within an organism, it would seem logical to predict that any unusual perturbation of intercellular communication could lead to multiple dysfunctional physiological conditions leading to various diseases (6). It has been postulated that teratogenesis (7), carcinogenesis (2), specifically the tumor promotion (8) and metastatic phases (9), neurotoxicity (10) and reproductive dysfunction (6) might be related to altered intercellular communication.

Gap junctional intercellular communication: Roles in the regulation of control of normal cell proliferation and function. The gap junction, a collection of closely packed pairs of transmembrane channels (the connexons), allows ions and low molecular weight molecules to diffuse from a cell to its neighboring cells (11). The connexon appears to consist of few homologous protein subunits (12).

The role of gap junctions has been linked to the control of cell proliferation in mitotic cells, leading to the phenomenon of "contact inhibition," to the control of differentiation in stem or progenitor cells; and to the control of differentiated functions in postmitotic cells (13-15). The coupling of homologous premitotic cells in a tissue would allow a means to synchronize function and to maintain an equilibrium of regulatory ions/molecules below a certain level. By inhibiting gap junctional communication, each cell becomes an automous unit. Therefore, molecular signals, such as mitogens, by inhibiting gap junctional intercellular communication, could allow critical regulatory ions and molecules to exceed "critical mass" levels to trigger the cell to enter a new physiological state (6,16,17). If gap junctional communication in tissues exposed to mitogens could not be inhibited, the mitogen-induced transmembrane signals to trigger mitogenesis would be diluted out by diffusion, preventing a critical mass level needed for conversion of a quiescent cell to a mitogenic state.

The mechanism controlling the interaction of the systemic form of intercellular communication and the local or gap junctional form, although not well understood, seems to provide a perfect cybernetic, homeostatic control system, by which both positive and negative growth factors can affect intracellular levels of regulatory ions/molecules which are capable of being transferred via gap junctions. The fact that normones and neurotransmitters can modulate gap junctions serves as examples of this coordinated means of intercellular communication (18,19).

Abnormal modulation of gap junction and dysfunctional intercellular communication. Recognizing the fact that chemical modulation of gap junction-mediated intercellular communication does occur, presupposes an understanding of the basic mechanisms by which this form of intercellular communication is regulated. Conceptually, there are several basic steps needed for cells to have functional gap junctional intercellular communication; namely, (a) cell adhension, possibly requiring cell-adhension molecules ("CAMs"); (b) formation of functional gap junctions from the hemi-units or connexions; (c) transfer of regulatory ions and small molecules; and (d) transduction of these regulatory signals into a transition of intracellular physiological states (20).

The demonstration that antibodies to cell surface cell adhension molecules can inhibit gap junctional intercellular (21), and that some cells can selectively communicate among themselves but not with heterologous cells (22), supports the idea that interference with nongap junctional components of a cell can affect this form of cell communication. In addition, cells with reduced gap junctional communication are usually associated with the inability to contact inhibit (13) or to be tumorigenic (2,14).

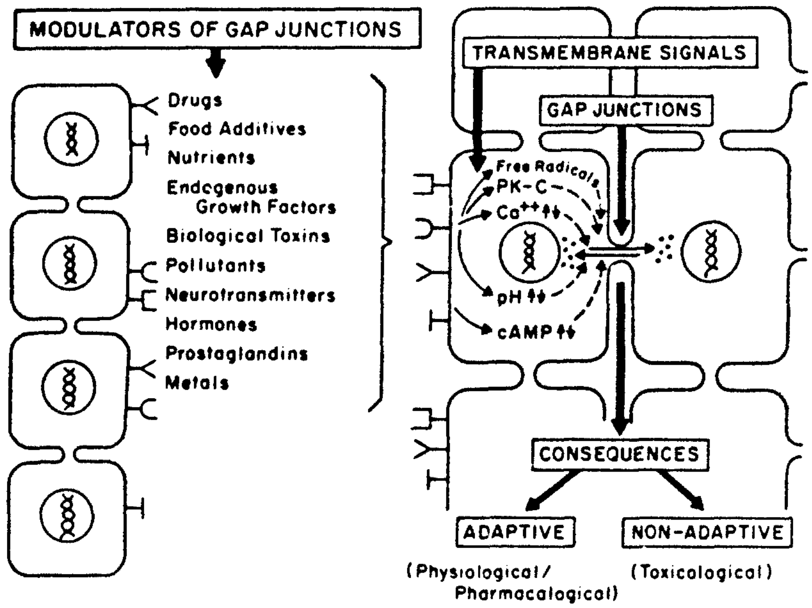

FIG. 12-2. This diagram summarizes how a variety of chemicals might modulate gap junction function by receptor or non-receptor mediated second messengers or by other mechanisms. The consequences of either decreasing or inducing gap junctional communication will depend on the circumstances (e.g., compensatory hyperplasia leading to either wound healing or to tumor promotion). (From Trosko *et al.* (1988), with permission from Princeton Scientific Publishing, Inc.).

The biochemical mechanism(s) by which gap junctional function is (are) modulated are only now becoming elucidated. Intracellular modulation of several "second messages," as well as membrane perturbations, seem to have been linked to alteration in gap junction structure/function. Increases in intracellular free Ca^{++} (23), modulation of the cytoplasmic pH (24), activation of protein kinase C dependent phosphorylation of the gap junction protein (25), activation of C-AMP-dependent protein kinase (26) and possible free radical damage of plasma membrane or of the gap junction protein structure (27) have been suggested mechanisms by which various endogenous and exogenous chemicals might modulate gap junction function (Fig. 12-2).

ROLE OF INHIBITED GAP JUNCTIONAL COMMUNICATION IN CARCINOGENESIS

Cancer as a stem-cell disease or the "blocked ontogeny" hypothesis. One of the earlier descriptions of cancer has been that of cancer as a "disease of differentiation" (1). If one assumes that the differentiated cell is derived from a stem or progenitor cell, then the hypothesis that carcinogenesis is the process by which a genetic block (mutation) has interfered with a stem cell's ability to terminally differentiate in response to a signal, sets the stage for suggesting that gap junctional communication might be involved.

Recent studies have suggested that some normal human fetal kidney epithelial cells do not seem to have functional intercellular communication (14). Under normal conditions, these cells must be held in check by some negative inhibitory factors, possibly coming from a differentiated daughter cell of that lineage (28). If these stem-like cells are exposed to a carcinogen which prevents its ability to terminally differen-

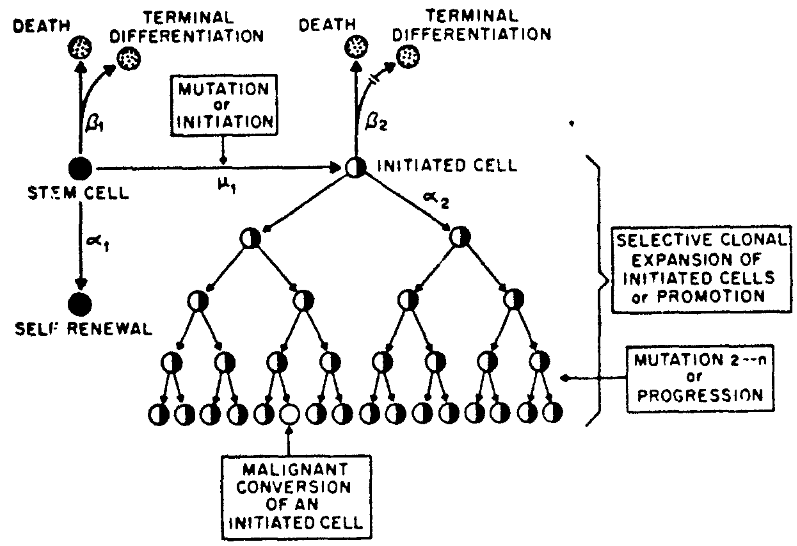

FIG. 12-3. The initiation/promotion/progression model of carcinogenesis. β_1=rate of terminal differentiation and death of stem cell; β_2=rate of death, but not of terminal differentiation of the initiated cell (SEE COPY). α_1= rate of cell division of stem cells; α_2=rate of cell division of initiated cells; μ_1=rate of the molecular event leading to initiation (i.e., possibly mutation); μ_2=rate at which second event occurs within an initiated cell. (From Trosko *et al.* (1988), with permission from Alan R. Liss, Inc.).

tiate after exposure to a factor, yet retain its ability for self-renewal (i.e., its ability to respond to positive growth factors), it has the potential to clonally amplify into a large mass of nondifferentiated, and therefore dysfunctional but not malignant, cells. Where in the differentiation pathway the block has occurred will determine the degree of differentiation shown by the tumor (1). The ability of many natural differentiation factors or exogenous chemicals to induce tumor cell to differentiate is consistent with this view of carcinogenesis (14) (Fig. 12-3).

The initiation/promotion/progression concept of carcinogenesis. Carcinogenesis seems to consist of several distinct phases in both natural and experimental carcinogenesis (29). The initiation phase of carcinogenesis appears to explain how a single stem cell, after exposure to mutagenic agents, is irreversibly prevented from terminally differentiating. Experimentally, initiation of mouse skin keratinocytes has been shown to induce the production of terminal-differentiation-deficient cells (30-33). Promotion is defined as the process to increase the earlier appearance and frequency of tumors in animals exposed to a carcinogen (i.e., initiator). On the cellular level, promotion would be acting as a mitogen to cause the selective accumulation of the initiated cells. Progression is then defined as the conversion of one of these cells in the promoted clone of initiated (but not yet malignant) cells to acquire the phenotypic feature needed to become malignant.

Chemical tumor promoters as inhibitors of intercellular communication. By definition, tumor promoters are those chemicals or conditions (i.e., wounding, necrosis [34]) which can give the initiated cell a chance to clonally amplify (8). Therefore, they are mitogens. In fact, many tumor promoting chemicals, such as the phorbol esters, do enhance proliferation of some cell types and induce differentiation of other cell types (35). The phorbol esters (and other tumor promoters) were shown to inhibit

gap junctional communication, in a reversible fashion, in *in vitro* systems (36,37), and to reduce gap junction numbers both *in vitro* (38) and *in vivo* (39,40). It may, therefore, be hypothesized that tumor promoters, by inhibiting gap junction function between an initiated cell and its surrounding normal cell, allowed the initiated cell to escape the mitotic-suppression of the normal cells (8,36). Subsequently, many different classes of tumor promoting chemicals, such as phenobarbital, DDT, polybrominated biphenyls, unsaturated fatty acids and saccharin could inhibit gap junctional intercellular communication (41).

Oncogenes as modulators of gap junctional intercellular communication. Oncogenes have been defined as sequences of DNA information with a proven cancer association that appear to function primarily in the regulation of cellular proliferation and differentiation (42). The programmed expression of several normal cellular oncogenes has been associated with the processes of normal cell proliferation, cell differentiation or the induction of differentiated functions in postmitotic cells (43,44). Oncogenes have been classified by the protein products for which they code. Some code for growth factors, growth factor receptors, transmembrane-signaling elements, as well as nuclear associated proteins (45).

Many activated oncogenes have been associated with tumors (46), and in particular, the ras-oncogene has been associated with metastatic growth (47–51). It would appear that there might be an association with certain oncogenes and the inability to terminally differentiate, to contact inhibit and to metastasize.

Since it is known that most tumor cells do not perform functional gap junctional intercellular communication (i.e., they are selective communicators or universally non-communicators [22,52]), it was not surprising that communication-competent cells, transfected with various oncogenes (e.g., V-src, ras, mos) (53–56) or the polyoma adenovirus middle T gene (57), were transformed to noncommunicating or selective communicating cells. It is noteworthy that some expressed oncogenes, which act as growth or differentiation inducers, have similar effects on cells as do some known tumor promoters such as the phorbol esters, which inhibit gap junctional intercellular communication.

Growth factors and the inhibition of gap junctional intercellular communication. Growth factors, by definition, are those cellular products which can induce a quiescent cell, which is mitogenically-suppressed by a negative growth factor either by direct gap junction dependent contact or by a diffusable mechanism, to enter into a synthetic phase which leads to cell proliferation. Since the tumor promotion phase of carcinogenesis involves, among other things, the clonal proliferation of a suppressible initiated stem-like cell, chemical tumor promoters, which inhibit gap junctional intercellular communication in a reversible manner, can be thought of as growth factors for the initiated cell. Therefore, one would predict that natural growth factors would be able to inhibit gap junctional intercellular communication in contact-inhibited cells. Recently, several growth factors, such as the epidermal growth factor (EGF) and the transforming growth factor-beta, (TGF-β), have been shown to block intercellular communication in normal human keratinocytes (58).

In a manner similar to the phorbol ester promoters and several oncogenes, several growth factors can have divergent effects on certain cell types, in that they can either induce proliferation or differentiation (59–61). In addition, some growth factors have been shown to have tumor-promoting-like activities, *in vitro* and *in vivo* (62–66).

INHIBITED INTERCELLULAR COMMUNICATION: A COMMON MECHANISM LINKING CHEMICAL TUMOR PROMOTERS, ONCOGENES AND GROWTH FACTORS

Chemical tumor promoters do act as growth factors for initiated cells; oncogenes code for growth-related polypeptides and growth factors have been shown to act as tumor promoters. While the specific mechanism by which any molecules of each of these three classes act to trigger mitogenesis can be quite different, the shared effect is their ability to inhibit gap junctional intercellular communication. Consequently, a chemical tumor promoter might trigger protein kinase C activity; this, in turn, via a series of phosphorylation reactions, could (a) prepare the plasma membrane for active transport of regulatory ions and substrates for macromolecular synthesis; (b) activate/inactivate pre-existing enzymes in the quiescent cell; (c) inhibit gap junctional intercellular communication; and (d) induce new gene expression for the mitogenic process.

Oncogenes, coding for growth factors or growth factor receptors, might work through another pathway, not needing PKC. Yet, they, too, would need to inhibit gap junctional intercellular communication in order to prevent critical levels of mitogen-triggering ions or chemicals from being drained to neighboring cells.

Unlike chemical tumor promoters, which need to be present at a high enough level (67-69) and for regular and chronic exposures (70), an oncogene, once expressed, would be a relatively stable inhibitor. Growth factors, once expressed, would be a constant stimulus either during scheduled phases of growth or periods of regeneration. This might explain the relative ease of inducing carcinogenesis in young animals or the relatively high risk young children have with several kinds of cancer.

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